Environmental Fate and Transport of Veterinary Antibiotics

Jonathan Charles Callura

ABSTRACT

Veterinary antibiotics such as ionophores and tetracyclines are commonly used in farming operations to prevent disease and promote growth rates in poultry, cattle, and swine. Since waste products from treated animals are used as fertilizers, there is a growing concern that these compounds may leach into the soil and water supply causing chronic low-level exposure to humans and leading to the development of antibiotic-resistant bacteria. Due to close structural similarities and physical properties, nigericin was determined to be an effective surrogate standard for the detection of the monensin, salinomycin, and narasin. Aluminum sulfate addition was considered as a potential treatment method to reduce antibiotic mobility, which resulted in an approximately 80% reduction of recovery rates for tetracyclines in poultry litter samples. Strong anion exchange cartridges were used in tandem with standard hydrophilic-lipophilic balanced cartridges which resulted in more rapid extractions and faster sample processing. Demeclocycline exhibited the potential to be used as a surrogate standard for tetracyclines due to close similarities in detection levels. This study has developed a method for detecting antibiotics in several types of environmental media and reinforced potential treatment methods as a means to reduce the risk of exposure to microcontaminants in the water supply.

1. INTRODUCTION

The implications of trace contaminants such as pharmaceutical products in environmental systems has been a cause for increasing concern in recent decades, stemming from detection of select compounds in natural systems. Veterinary antibiotics, traditionally applied as feed additives, are utilized to prevent disease and promote growth among livestock and poultry. One study suggests that this application accounts for 24.6 million pounds, or approximately 70% of total antimicrobial use in the United States (Mellon, Benbrook, & Benbrook, 2001). As the leading producer in the nation, the state of Georgia generated over 1.3 million broilers, the industry term used for chickens grown for the purpose of meat production, in 2010 (National Agricultural Statistics, 2011).

Bedding materials such as wood shavings from broiler operations are routinely mixed with feathers, wasted feed, and broiler excrement to produce poultry litter that is then used as a grassland fertilizer due to its nitrogen, phosphorus, and potassium content (Nichols, Daniel, & Edwards, 1994). Since up to 90% of the dosage passes directly through the animals' digestive systems and becomes incorporated into the litter, the antibiotics may potentially be leached into sediments and soil, as well as nearby surface and groundwater sources. A recent study of several large-scale swine and poultry feeding facilities noted that antimicrobial compounds were detected in 67% of surface and groundwater sources within close proximity to the farms (Campagnolo, et al., 2002). An illustration of the mobilization pathways may be seen in figure 1. One primary concern of the presence of such microcontaminants is the development of antibiotic-resistant bacteria which would compromise the efficacy of current treatment methods. Furthermore, much remains unknown about the toxicity associated with chronic low-level exposure to anthropogenic stressors.

Michael James

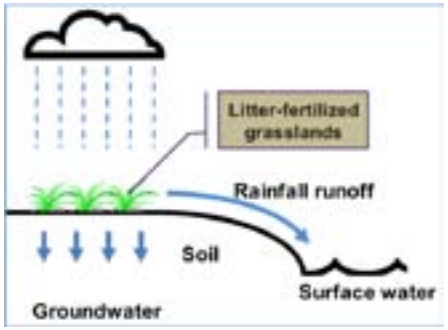

Figure 1. Depiction of antibiotic mobilization pathways.

Ionophores (IP) and tetracyclines (TC) are particularly relevant due to the frequency of their usage as anticoccidials in the poultry and livestock industries. The IP under review are monensin (MON), salinomycin (SAL), narasin (NAR), and nigericin (NIG), while observed TC include oxytetracycline (OTC), chlortetracycline (CTC), tetracycline (TTC), and demeclocycline (DMC).

Surrogate standards are compounds that are not expected to be present in environmental samples but have similar structure and properties to the target analytes and may indicate any unusual matrix effects. The first goal of this study is to further develop experimental methods and to validate the selection of NIG as a surrogate standard for IP. Aluminum sulfate (alum) may be applied to poultry litter prior to soil fertilization to decrease phosphorus runoff and ammonia volatilization (Moore, Daniel, & Edwards, 2000). Antibiotics are expected to exhibit reduced mobility when subjected to alum treatment due to decreased sample pH and potential Al-antibiotic complexation reactions. While recent studies have primarily employed the use of hydrophilic-lipophilic balanced (HLB) solid phase extraction (SPE) cartridges in their analyses, some literature suggests a strong anion exchange (SAX) and HLB tandem cartridge setup (Hansen, Bjorklund, Krogh, & Halling-Sorensen, 2009); (Blackwell, Lutzhoft, Ma, Halling-Sorensen, Boxall, & Kay, 2004). Another purpose of this project is to attempt to improve TC detection through the use of the SAX-HLB configuration and pH alteration.

2. METHODS

2-A. Rainfall Runoff Extraction

The first of the two primary analysis methods involves antibiotic detection from a liquid matrix. An experimental field at the University of Georgia (Athens, GA) was isolated and treated with poultry litter fertilizer. The area was covered in order to prevent mobilization from unexpected rains and subjected to a rainfall simulation from a spray tower and the surface runoff was collected in amber bottles for storage. The samples were then sent to the Georgia Institute of Technology (Atlanta, GA) for analysis and kept under refrigeration.

Liquid samples were measured to 200 mL volumes and spiked with antibiotics prior to being passed through a vacuum-powered 0.45 um glass fiber filter to remove excess suspended solids which may interfere with analysis. The samples were then adjusted to a pH of 6-7 spiked with IP and TC to a concentration of 1000 PPB. Disodium ethylenediamine tetraacetate (Na2EDTA) was added to a concentration of 1 mM as a buffer and the samples were passed through HLB extraction cartridges under vacuum pressure. Once fully passed, the cartridges were washed with 5 mL of deionized water and dried to prepare them for elution. Two 3 mL quantities of methanol (MeOH) were slowly passed through the cartridges after a soaking period of 5 minutes and the contents were collected in glass vials beneath the vacuum manifold.

2-B. Soil and Poultry Litter Extraction

For solid phase experiments, samples were weighed out to 1 g and spiked directly with antibiotics before being spread on plastic wrap to dry for 2-3 hours. Samples from the alum addition experiments were also spiked with 0.2 g of finely ground aluminum sulfate powder. Once dry, the soil or poultry litter was transferred to centrifuge tubes and mixed with an extraction solution whose contents are shown in tables 1, 2, or 3 depending on the method used.

Table 1. Standard Extraction Solution

McIlvain's Buffer at pH 7 with 10 mM Na2EDTA (mL)	MeOH (mL)	Total Volume (mL)
10	10	20

Table 2. Method 1 Extraction Solution (pH = 3)

0.1M Citric Acid (ml)	0.2 M Na2H-PO4 (ml)	10mmol Na2EDTA solution (ml)	DI water (ml)	MeOH (ml)	Total Volume (ml)
7.95	2.05	2	3	5	20

Table 3. Method 2 Extraction Solution (pH = 6)

0.1M Citric Acid (ml)	0.2 M Na2H-PO4 (ml)	10mmol Na2EDTA solution (ml)	DI water (ml)	MeOH (ml)	Total Volume (ml)
3.68	6.32	2	3	5	20

The tubes were shaken for 30 minutes at 400 RPM on an agita-

tion table then placed on a centrifuge machine for 10 minutes at 5000 RPM. The 20 mL of supernatant fluid from the centrifuge process was separated and diluted with 460 mL of water before another batch of extraction solution was added to the centrifuge tube and shaken once more. The supernatant fluid was separated again, bringing the final diluted volume to 500 mL. Next, the SPE process was conducted as outlined in the water sample section, with the aforementioned SAX-HLB tandem cartridges used in the alum addition experiments.

2-C. Clean-Up Steps

Fluids from the SPE process were blown to dryness under vacuum then mixed with 5 mL ethyl acetate and 0.5 mL 0.1 M NaCl to separate analytes from impurities. The resulting dirty water layer was pipetted out of the vial and the samples underwent vacuum volume reduction once more. The vials were then injected with equal parts MeOH and 0.1 M NaCl as well as 25 uL of simatone, an internal standard, and taken to be analyzed on liquid chromatography-mass spectrometry (LC-MS) equipment which utilizes electrospray ionization (ESI) and selected ion monitoring (SIM) to effectively determine sample composition. Figures 2 and 3 illustrate overviews for the extraction procedures for liquid and solid matrices, while figure 4 shows the clean-up procedures in detail.

3. RESULTS

3-A. Surrogate Standard Validation

To validate the applicability of NIG as a surrogate standard for the selected compounds, a series of spiked samples were processed from all relevant matrices and the peak areas were plotted with their corresponding concentrations.

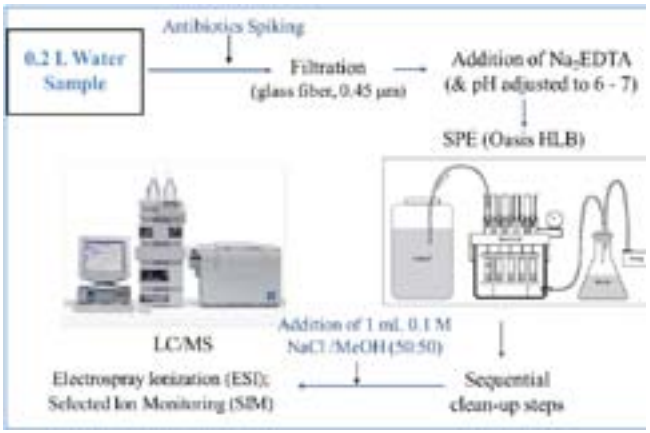

Figure 2. Water sample extraction process.

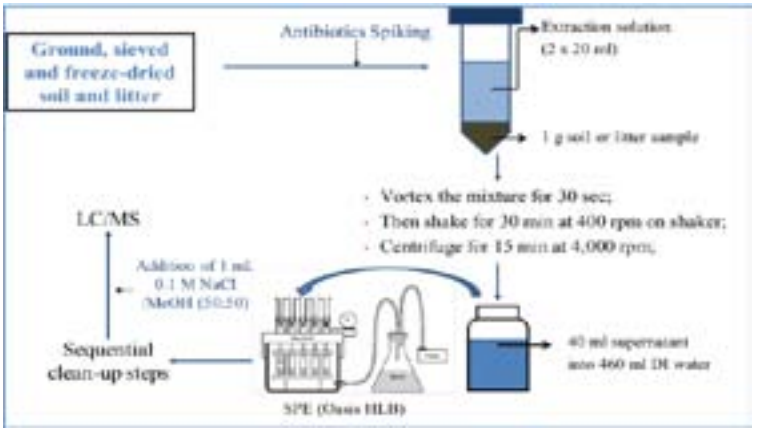

Figure 3. Soil and poultry litter sample extraction process.

Figure 4. Sequential clean-up process.

Figure 5 shows peak area readings from the LC-MS software. The unitless value is related to the solution concentration. The spiked concentrations are based off a calibration curve obtained from samples of known concentrations. This data was then normalized using NIG as the denominator for both values, producing the information shown in figure 6. Figure 7 shows normalized data for SAL soil samples and NAR spiked rainfall runoff samples.

3-B. Aluminum Sulfate Addition

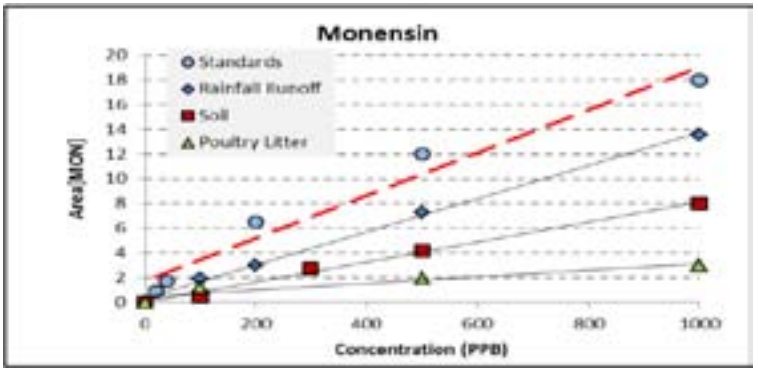

Figure 5. MON peak area vs. concentration for various media.

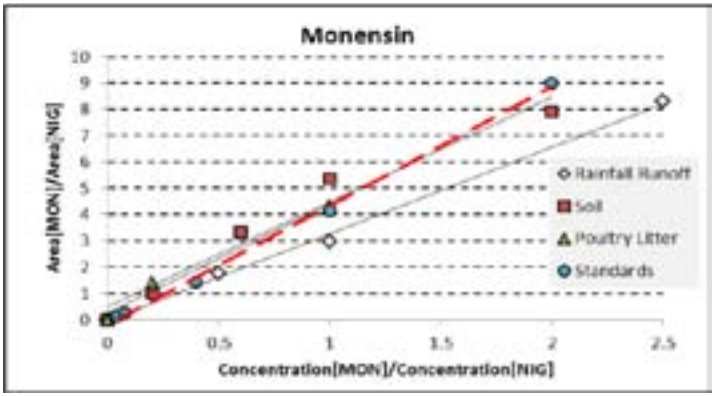

Figure 6. MON normalized area vs. normalized concentration.

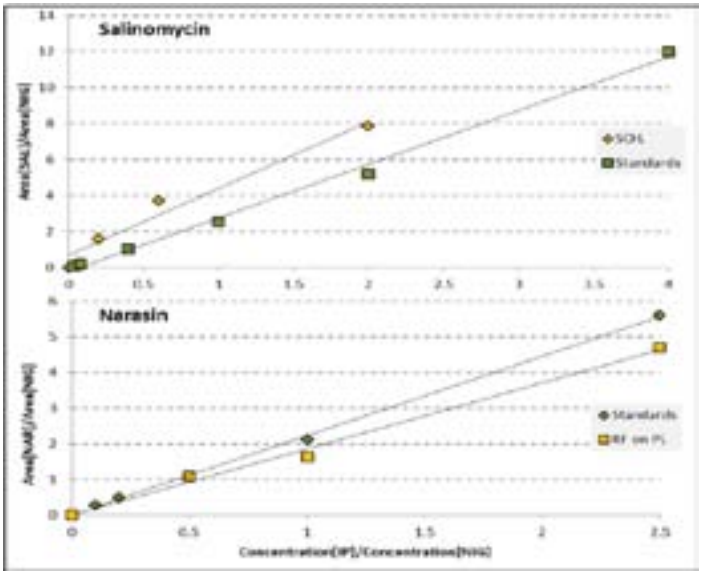

Figure 7. SAL and NAR normalized area vs. normalized concentration.

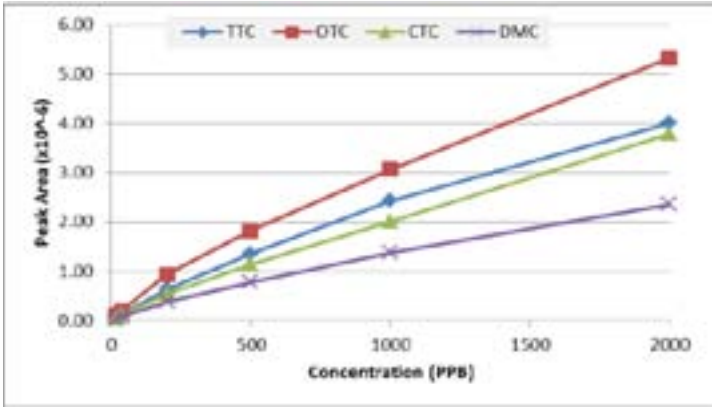

Figure 8. TC calibration curve.

Calibration curves for each compound were created by preparing standard solutions of 20, 40, 200, 500, 1000, and 2000 PPB as shown in figure 8. A linear regression was performed for the curves and the resulting second-order polynomial equations were used calculate concentrations for the poultry litter samples. Three different methods were used in preparing the samples: method 1, method 2, and alum addition. Method 1 followed the standard solid sample extraction steps using the 'Method 1' extraction solution, while method 2 utilized the other corresponding extraction solution. The supernatant fluid from method 2 was also injected with hydrochloric acid until a pH of 3 was reached. The 'Alum PL' sample followed standard solid extraction methods except 0.2 g of aluminum sulfate was added to the litter prior to spiking with antibiotics. Based on the calculated concentrations, recovery rates were determined according to the following equation with the results shown in figure 9:

$$\text{Recovery (\%)} = \frac{C_s - C_x}{S}$$

Where CS = concentration in spiked samples, CX = concentration in unspiked samples, & S = spiking concentration

4. DISCUSSION

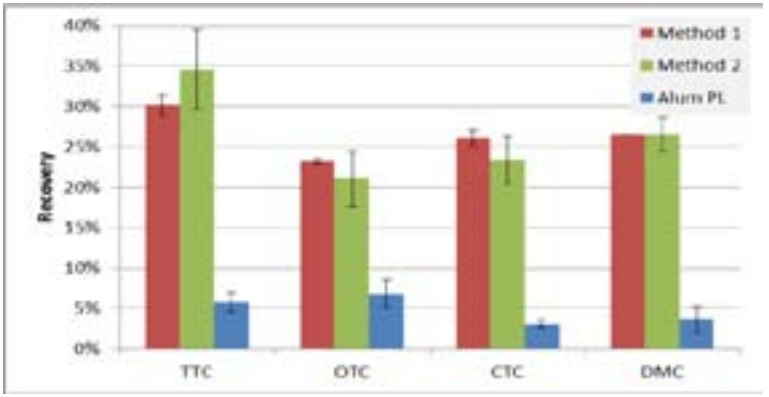

Figure 9. TC recovery rates for alum poultry litter and regular spiked poultry litters.

4-A. Surrogate Standard Validation

The relationships developed by the data in figures 5-7 show a correlation between the amount of NIG and the amount of other IP detected in a sample. The differences between figures 5 and 6 illustrate the benefits of NIG, as evident by the convergence of all samples around the standard line which accounts for variance based on the matrix type. Based on this connection, we may use NIG as an indicator of data quality since extreme deviations from the observed results may suggest errors or interference. Nigericin was determined to be a suitable surrogate standard for the

selected ionophores which supports the proposed hypothesis.

4-B. Aluminum Sulfate Addition

The observed recovery rates for tetracyclines in this study (figure 9) followed hypothesized behavior, resulting in a significant mobility reduction of samples with aluminum sulfate. DMC exhibited the potential to be used as a surrogate standard for TC detection based on the similarities in recovery rates with the other TC species. Methods 1 and 2, differing only in timing of pH reduction, each reported recovery rates of approximately 25%, compared to less than 5% for alum samples. These values account for the negligible existing levels of TC in blank samples and are likely below 50% due to the particularly prevalent matrix interference and signal suppression in soil and poultry litter. It is important to note that without any sort of pH alteration, TC were not detected in solid matrices so all samples were acidified. Method 1 was expected to yield slightly higher recovery rates due to acidification of the matrix which creates a stable environment for tetracyclines, but only marginal improvements were noted when compared to method 2. Method 2 samples were extracted with a neutral pH extraction solution and later the supernatant fluids from the centrifuge process were acidified. Although the SAX-HLB cartridge setup marginally increased detection when compared to earlier experiments, the greatest benefit appeared to be improvements in extraction speed. Since the SAX cartridges capture humic acid and natural organic matter which is present in both soil and poultry litter, they prevented clogging and shortened the time required for processing.

5. CONCLUSIONS

This study develops a method for determining the fate and mobility of antibiotics in several compartments of the environment. By validating the selection of nigericin as a surrogate standard for ionophores, more effective analyses may be conducted in the future. Analysis of tetracyclines in various environments highlighted the need for pH reduction to aid in detection. Perhaps most importantly, the restriction of antibiotic mobility, which occurred as a result of aluminum sulfate addition to soil and litter, provides a potential means for pollution prevention as it is already a common practice in preventing nutrient runoff. By mixing alum treatment with litter fertilizers, farming operations may deter the transport of microcontaminants into surface runoff and groundwater sources, effectively limiting exposure levels to the public water supply. This work provides groundwork for further experimental development by future researchers in the rapidly developing field of environmental antibiotic contaminant study.

Future Work

In order to fully understand the transport of ionophores and tetracyclines, further studies must be conducted. The effects of each matrix on analyte detection and the corresponding signal suppression must continue to be evaluated. While NIG was thoroughly validated as a surrogate for MON, similar experiments must be conducted for the remaining matrices associated with SAL and NAR. Finally, the effects of stacking and storage methods on compound detection must be understood.

6. REFERENCES

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